PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 37/02, C07K 5/00, 7/00, 15/00, 17/00 — A1

(11) International Publication Number: WO 94/26296

(43) International Publication Date: 24 November 1994 (24.11.94)

(21) International Application Number: PCT/US94/05616

(22) International Filing Date: 18 May 1994 (18.05.94)

(30) Priority Data:
08/064,517 19 May 1993 (19.05.93) US

(71) Applicant: HOUGHTEN PHARMACEUTICALS, INC. [US/US]; Building 2, Room 138, 3550 General Atomics Court, San Diego, CA 92121 (US).

(72) Inventors: DOOLEY, Colette, T.; 1735 Reed Avenue, #4, San Diego, CA 92109 (US). HOUGHTEN, Richard, A.; 4939 Rancho Viejo, Del Mar, CA 92014 (US).

(74) Agents: KONSKI, Antoinette, F. et al.; Campbell & Flores, Suite 700, 4370 La Jolla Village Drive, San Diego, CA 92122 (US).

(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: NOVEL OPIOID PEPTIDE INHIBITORS

(57) Abstract

This invention provides novel peptides having the ability to inhibit binding of the mu specific ligand ^{3}H-[D-Ala^{2}, MePhe^{4}, Gly-ol^{5}] enkephalin ("DAGO") to the opioid receptors in crude rat brain homogenate.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

NOVEL OPIOID PEPTIDE INHIBITORS

BACKGROUND OF THE DISCLOSURE

This invention generally relates to novel peptides having the ability to inhibit ligand binding to an opioid receptor.

There are at least three known subtypes of opioid receptors, mu, kappa, and delta; with some evidence for two additional receptor subtypes. The use of synthetic peptides has been instrumental in the delineation of these subtypes and for providing analogues that can be used for studying the interactions of ligands specific to these receptor systems in both in vitro and in vivo systems.

Recent advances in methods for the preparation and screening of a large numbers of individual peptides has enabled a large number of peptides to be used in all areas of biomedical research, including research regarding the interaction of a ligand to the opiate receptor. Even with these advances, however, basic research and drug discovery has been limited by the availability of the requisite large number of diverse opiate agonists and antagonists required to ascertain the relationship between a ligand for a particular opiate receptor subtype. Thus, a need exists for large numbers of individual peptides for use in biomedical research, including those for the study of opiate ligand-receptor interactions. This invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

This invention provides novel peptides having the ability to inhibit ^{3}H-[D-Ala^{2},MePhe^{4},Gly-ol^{5}]enkephalin ("DAGO") binding to opioid receptors in crude rat brain homogenates. The novel peptides fall within four general structures: Ac-L-Arg-L-Phe-L-Met-L-Trp-L-Met-L-Thr-L-Xaa-NH_2

(SEQ ID NO: 1); Ac-D-Arg-D-Phe-D-Trp-D-Trp-D-Gly-D-Xaa-NH_2 (SEQ ID NO: 2); Ac-D-Arg-D-Phe-D-Trp-D-Ile-D-Asn-D-Xaa-NH_2 (SEQ ID NO: 3); and Ac-D-Arg-D-Phe-D-Trp-D-Met-D-Tyr-D-Xaa-NH_2 (SEQ ID NO: 4). Within each genus, Xaa is substituted by a specific amino acid.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A through 1D graphically depict the ability of each peptide to inhibit binding of [^{3}H]-DAGO to the μ receptor as measured by the radio-receptor assay. In these figures, "o" is the equivalent of the amino acid code "Xaa".

DETAILED DESCRIPTION OF THE INVENTION

This invention provides peptides useful as inhibitors of ^{3}H-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin ("DAGO") which is known to bind with high selectivity to the μ ("mu") opioid receptor subtype. Each of the peptides has an acetyl group ("Ac") at the N-terminus and an amide group ("NH_2") on the C-terminus. In one embodiment, the peptide has the structure Ac-L-Arg-L-Phe-L-Met-L-Trp-L-Met-L-Thr-L-Xaa-NH_2, wherein Xaa is an L-amino acid such as L-Lys, L-Arg, L-Thr, L-Ser, L-Gln, L-Pro, L-His, L-Ala, L-Asn, L-Met, L-Gly, L-Leu, L-Tyr, L-Val, L-Trp, L-Asp, L-Cys, L-Glu, L-Phe, and L-Ile. In another embodiment, the peptide is described by the formula Ac-D-Arg-D-Phe-D-Trp-D-Trp-D-Gly-D-Xaa-NH_2, wherein D-Xaa is D-Leu, D-Met, D-Val, D-Trp, D-Lys, D-Tyr, D-Arg, D-Ile, D-Pro, D-Phe, D-His, D-Ser, D-Cys, D-Asn, D-Thr, D-Gln, D-Ala, D-Gly, D-Asp, or D-Glu. Also within the scope of this invention is a peptide having the structure Ac-D-Arg-D-Phe-D-Trp-D-Ile-D-Asn-D-Xaa-NH_2, wherein D-Xaa is a D-amino acid such as D-Lys, D-Ala, D-Arg, D-Gln, D-Pro, D-Asn, D-Ser, D-Tyr, D-Met, D-Gly, D-Thr, D-His, D-Trp, D-Leu, D-Phe, D-Glu, D-Cys, D-Val, D-Asp and D-Ile. Further provided by this invention is a peptide

having the structure Ac-D-Arg-D-Phe-D-Trp-D-Met-D-Tyr-D-Xaa-NH_2, wherein D-Xaa is a D-amino acid such as D-Arg, D-Lys, D-His, D-Ser, D-Thr, D-Gln, D-Pro, D-Ala, D-Asn, D-Gly, D-Tyr, D-Leu, D-Met, D-Phe, D-Cys, D-Trp, D-Glu, D-Asp, D-Val, or D-Ile.

One skilled in the art, using the above formulae, can easily reproduce the peptides of this invention by synthesis on an automated peptide synthesizer (Model 430A, Applied Biosystem, Foster City, California USA) utilizing the directions provided by the manufacturer. After manufacture, the peptides are assayed for receptor binding activity using the radio-receptor assay outlined below. Because these peptides bind to the μ and other receptor subtypes, they can be used in <u>in vitro</u> assays to study the opiate receptor subtypes. For example, in a sample receptor of unknown type or origin, the peptides, after being labeled with a detectable marker such as a radioisotope, can be contacted with the receptor sample under conditions which specifically favor a particular receptor subtype binding to the peptide(s). Unbound receptor and peptide can be removed, for example, by washing with a saline solution, and bound receptor can then be detected using methods well known to those skilled in the art.

In addition to the peptides utility in <u>in vitro</u> screening method for assaying organic compounds having specificity for the opioid receptors, the peptides also are useful as drugs to treat pathologies associated with other compounds which interact with the opioid receptor system. It can be envisioned that these peptides can be used for therapeutic purposes to block the peripheral effects of existing centrally acting pain killers such as morphine. Since it is known that the majority of peptides do not readily cross the blood-brain barrier (and therefore elicit no central effect), and since morphine has a number of

deleterious effects in the periphery which are not required for the desired analgesic effects, it can be anticipated that the subject peptides may have value in blocking the constipation and pruritis (itching) associated with morphine. The novel peptides claimed can be incorporated into pharmaceutical compositions. The pharmaceutical composition is prepared by combining the peptide with a pharmaceutically acceptable carrier. As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents.

Methods of administering a pharmaceutical are well known in the art and include but are not limited to administration orally, intravenously, intramuscularly or intraperitoneal. Administration can be effected continuously or intermittently and will vary with the subject and is dependent on the type of treatment and potency of the peptide used.

Radio-receptor assay

Crude membrane homogenates were prepared using a modification of the method described by Paternak, G.W. et al., Mol. Pharmacol., 11:340-351 (1975), incorporated herein by reference. Rat brains frozen in liquid nitrogen were obtained from Rockland, Inc. (Gilberstville, PA). The brains were thawed the cerebella removed, and the remaining tissue weighed. Each brain was individually homogenized in 40 ml Tris-HCl buffer (50mM, pH 7.4, 4°C) and centrifuged (Sorvall RC5C SA-600 16,000 rpm) for 10 minutes. The pellets were resuspended in fresh Tris-HCl buffer and incubated at 37°C for 40 minutes. Following incubation, the suspensions were centrifuged as before, the resulting

pellets resuspended in 100 volumes of Tris buffer, and the suspensions combined. Membrane suspensions were prepared and used in the same day. Protein content of the crude homogenates ranged from 0.15 to 0.2 mg/ml as determined using the method described by Bradford, M.M. Anal. Biochem. 72:248-254 (1976), incorporated herein by reference.

Binding assays were carried out in polypropylene tubes. Each tube contained 0.5 ml of membrane suspension, 8nM[^{3}H]-DAGO (specific activity 36Ci/mmole, 160,000 cpm), 0.08 mg/ml peptide mixture and Tris-HCl buffer in a total volume of 0.65 ml. Assay tubes were incubated for 60 minutes at 25°C. The reaction was terminated by filtration through GF-B filters. The filters were subsequently washed with 6 ml Tris-HCl buffer, at 4°C. Bound radioactivity was counted on an LKB Beta-plate Liquid Scintillation Counter and expressed in counts per minute (cpm). Inter- and intra-assay variation standard curves were determined by incubation of [^{3}H]-DAGO in the presence of a range of concentrations of unlabeled DAGO (0.13-3900nM). Both the tritiated and non-tritiated forms of DAGO were obtained from the National Institute of Drug Abuse (NIDA) repository, as prepared by Multiple Peptide Systems (San Diego, CA). A control curve was included on each plate for each assay (using a 96-well format). Competitive inhibition assays were performed as above using serial dilutions of the peptide mixture. IC_{50} values (the concentration necessary to inhibit 50% of [^{3}H]-DAGO binding) were then calculated using the software GRAPHPAD (ISI, San Diego, CA) and were found to be consistent in three determinations. The average values for each peptide are presented in Figure 1.

Although the invention has been described with reference to the disclosed embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly,

the invention is limited only by the following claims.

What is claimed is:

1. A peptide having the structure:

Ac-L-Arg-L-Phe-L-Met-L-Trp-L-Met-L-Thr-L-Xaa-NH_2;

wherein L-Xaa is an L-amino acid selected from the group consisting of L-Lys, L-Arg, L-Thr, L-Ser, L-Gln, L-Pro, L-His, L-Ala, L-Asn, L-Met, L-Gly, L-Leu, L-Tyr, L-Val, L-Trp, L-Asp, L-Cys, L-Glu, L-Phe, and L-Ile.

2. A peptide having the structure:

Ac-D-Arg-D-Phe-D-Trp-D-Trp-D-Gly-D-Xaa-NH_2;

wherein D-Xaa is a D-amino acid selected from the group consisting of D-Leu, D-Met, D-Val, D-Trp, D-Lys, D-Tyr, D-Arg, D-Ile, D-Pro, D-Phe, D-His, D-Ser, D-Cys, D-Asn, D-Thr, D-Gln, D-Ala, D-Gly, D-Asp, and D-Glu.

3. A peptide having the structure:

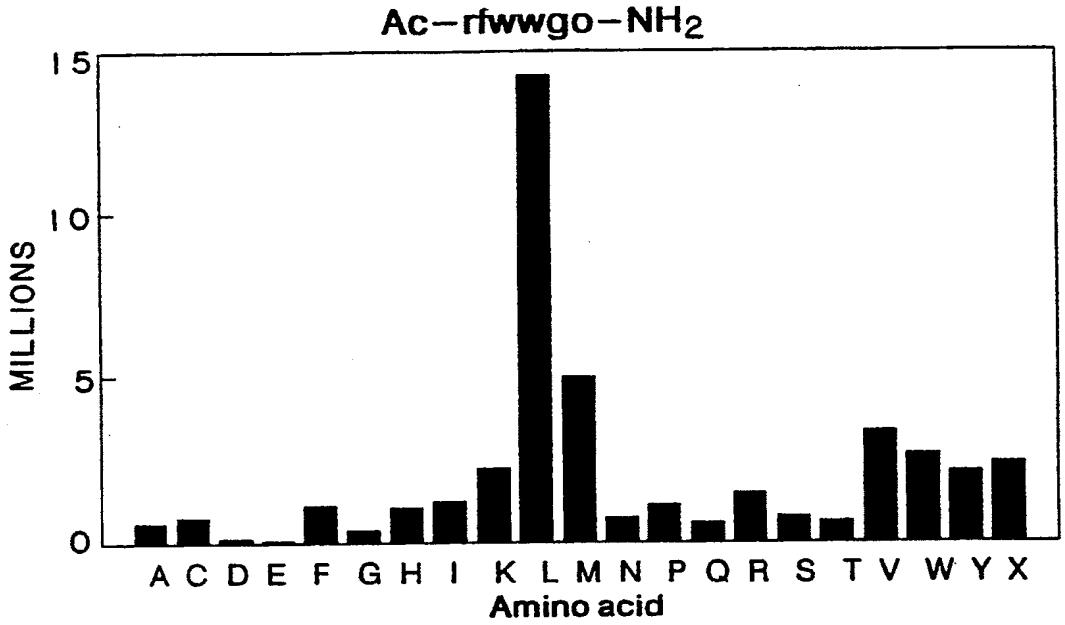
Ac-D-Arg-D-Phe-D-Trp-D-Ile-D-Asn-D-Xaa-NH_2;

wherein D-Xaa is a D-amino acid selected from the group consisting of D-Lys, D-Ala, D-Arg, D-Gln, D-Pro, D-Asn, D-Ser, D-Tyr, D-Met, D-Gly, D-Thr, D-His, D-Trp, D-Leu, D-Phe, D-Glu, D-Cys, D-Val, D-Asp and D-Ile.

4. A peptide having the structure:

Ac-D-Arg-D-Phe-D-Trp-D-Met-D-Tyr-D-Xaa-NH_2;

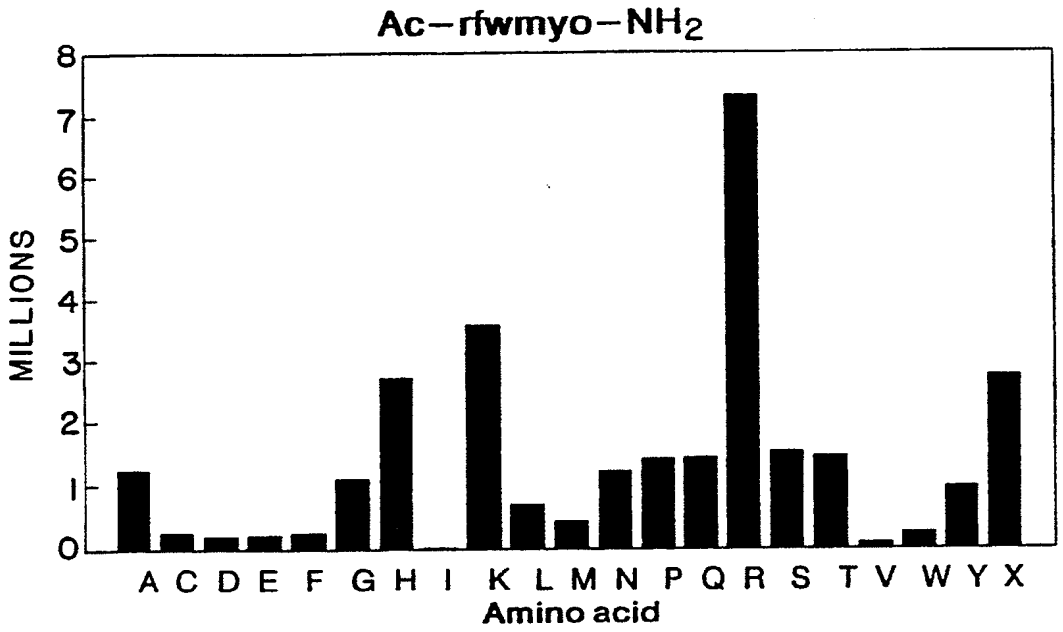
wherein D-Xaa is a D-amino acid selected from the group consisting of D-Arg, D-Lys, D-His, D-Ser, D-Thr, D-Gln, D-Pro, D-Ala, D-Asn, D-Gly, D-Tyr, D-Leu, D-Met, D-Phe, D-Cys, D-Trp, D-Glu, D-Asp, D-Val, and D-Ile.



Ac–rfwwgo–NH_2

	nM
Ac–rfwwgl	70
Ac–rfwwgm	201
Ac–rfwwgv	298
Ac–rfwwgw	377
Ac–rfwwgx	419
Ac–rfwwgk	451
Ac–rfwwgy	470
Ac–rfwwgr	671
Ac–rfwwgi	809
Ac–rfwwgp	871
Ac–rfwwgf	885
Ac–rfwwgh	923
Ac–rfwwgs	1283
Ac–rfwwgc	1295
Ac–rfwwgn	1370
Ac–rfwwgt	1584
Ac–rfwwgq	1665
Ac–rfwwga	1698
Ac–rfwwgg	2573
Ac–rfwwgd	8652
Ac–rfwwge	14855

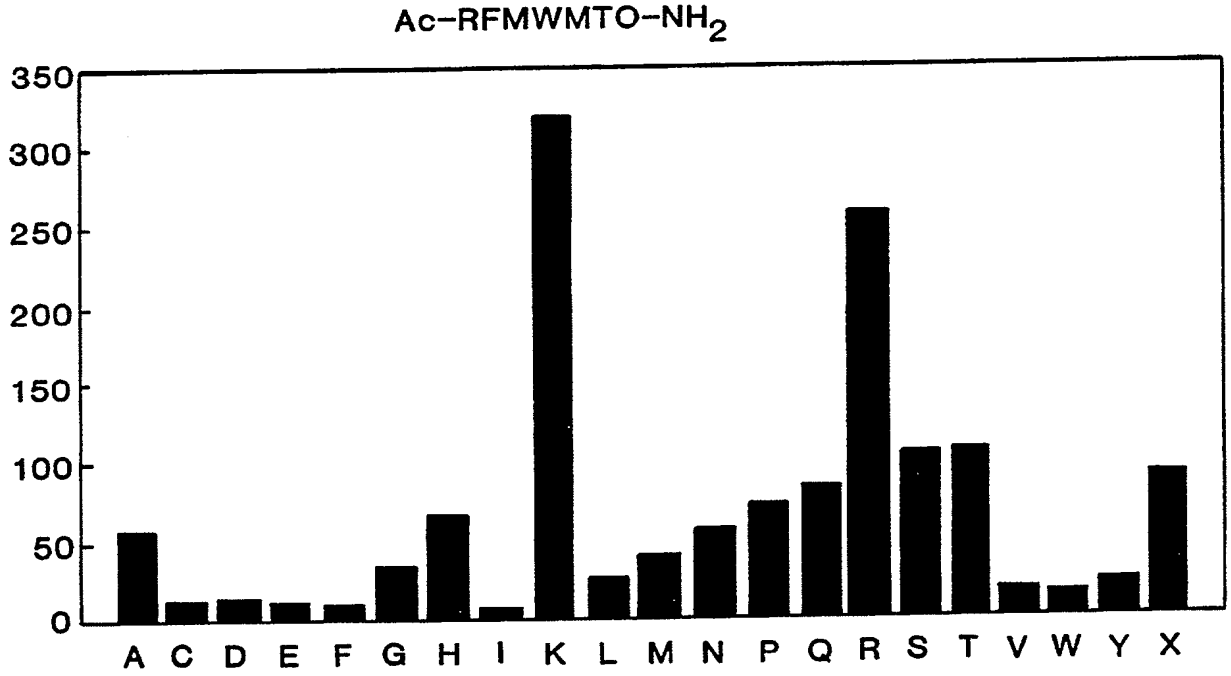
FIG. IA



Ac–rfwmyo–NH_2

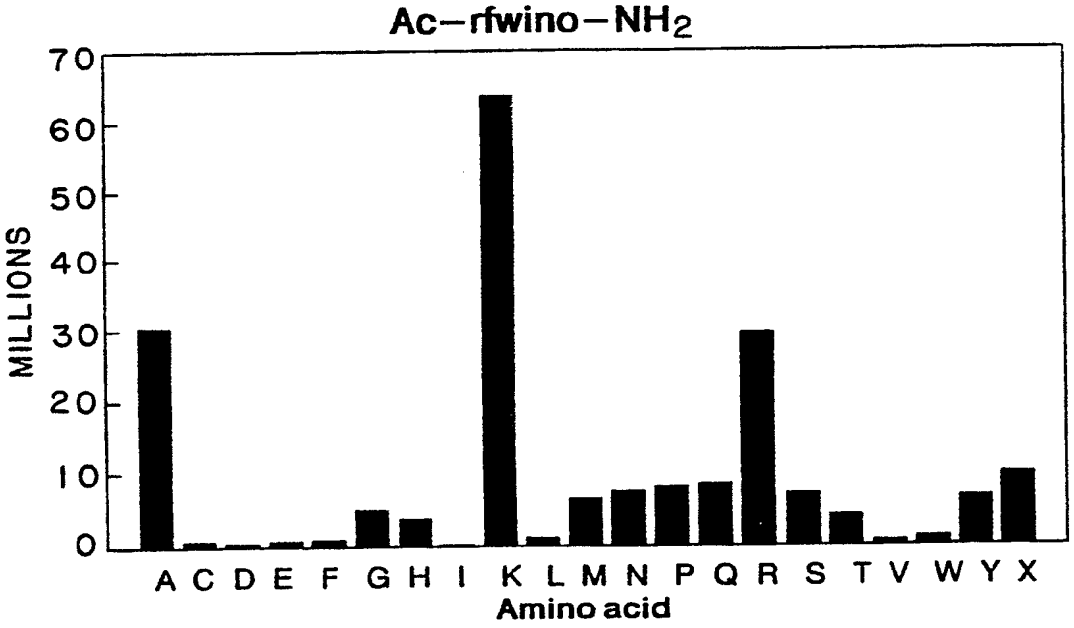
	nM
Ac–rfwmyr	137
Ac–rfwmyk	279
Ac–rfwmyx	366
Ac–rfwmyh	370
Ac–rfwmys	659
Ac–rfwmyt	697
Ac–rfwmyq	707
Ac–rfwmyp	720
Ac–rfwmya	816
Ac–rfwmyn	833
Ac–rfwmyg	922
Ac–rfwmyy	1044
Ac–rfwmyl	1457
Ac–rfwmym	2281
Ac–rfwmyf	3730
Ac–rfwmyc	3769
Ac–rfwmyw	3800
Ac–rfwmye	4496
Ac–rfwmyd	4980
Ac–rfwmyv	12402
Ac–rfwmyi	53641

FIG. IB



Ac-RFMWMTO-NH_2	nM
Ac-RFMWMTK	3
Ac-RFMWMTR	4
Ac-RFMWMTT	9
Ac-RFMWMTS	10
Ac-RFMWMT	11
Ac-RFMWMTQ	12
Ac-RFMWMTP	14
Ac-RFMWMTH	15
Ac-RFMWMTA	18
Ac-RFMWMTN	18
Ac-RFMWMTM	25
Ac-RFMWMTG	31
Ac-RFMWMTL	40
Ac-RFMWMTY	42
Ac-RFMWMTV	52
Ac-RFMWMTW	63
Ac-RFMWMTD	80
Ac-RFMWMTC	87
Ac-RFMWMTE	93
Ac-RFMWMTF	96
Ac-RFMWMTI	161

FIG. IC



Ac–rfwino–NH_2	nM
Ac–rfwink	16
Ac–rfwina	33
Ac–rfwinr	34
Ac–rfwinx	103
Ac–rfwinq	120
Ac–rfwinp	125
Ac–rfwinn	137
Ac–rfwins	142
Ac–rfwiny	155
Ac–rfwinm	159
Ac–rfwing	198
Ac–rfwint	244
Ac–rfwinh	277
Ac–rfwinw	826
Ac–rfwinl	897
Ac–rfwinf	1002
Ac–rfwine	1149
Ac–rfwinc	1311
Ac–rfwinv	1599
Ac–rfwind	1909
Ac–rfwini	8000

FIG. ID

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/05616

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/02; C07K 5/00, 7/00, 15/00, 17/00
US CL :530/328, 329

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/328, 329

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

USPTO APS
search terms: arg-phe-met-trp-met-thr, arg-phe-trp-trp-gly, arg-phe-trp-ile-asn, arg-phe-trp-met-tyr, dooley, houghten

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US,A, 4,396,606 (GOLDSTEIN) 02 August 1983.	1-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 19 JULY 1994	Date of mailing of the international search report AUG 02 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SHEELA J. HUFF Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*